Volume 314, number 1, 72–76 FEBS 11832 December 1992

Regulation of cardiac insulin receptor function by guanosine nucleotides

Martina Russ, Hans Reinauer and Jürgen Eckel

Laboratory of Molecular Cardiology, Diabetes Research Institute, 4000 Düsseldorf 1, Germany

Received 15 September 1992

The present study examined the effect of [^{35}S]GTP on the function of insulin receptors partially purified from adult rat cardiomyocytes by WGA chromatography. [^{35}S]GTP increased receptor autophosphorylation about two times and fully mimicked the stimulatory action of insulin on poly(Glu:Tyr) phosphorylation with no additional effect of the hormone. The effect of [^{35}S]GTP was specific, dose-dependent, and due to an increase in the V_{max} of the kinase. In the presence of ATP or AMP-PNP, insulin significantly enhanced the binding of [^{35}S]GTP to the partially purified insulin receptor. The findings suggest coupling of the insulin receptor to a G-protein which may be involved in the regulation of tyrosine kinase activity.

Isolated cardiac myocyte; Insulin receptor; Guanosine nucleotide binding protein; Tyrosine kinase

1. INTRODUCTION

In the past few years evidence has accumulated which suggests that insulin receptor signalling may involve coupling to GTP-binding proteins [1–7]. This view is mainly supported by the effects of cholera and pertussis toxin on insulin action. Thus, cholera toxin was found to modify insulin signalling in the liver [1] and in the heart [7], whereas pertussis toxin attenuates the activation of hexose transport and the generation of inositolglycan mediators in BC_3H-1 myocytes [3], and the antilipolytic activity of insulin in adipocytes [2]. More recently it was found that insulin increases GTP-binding to plasma membranes from fat and muscle cells [8,9] and that this binding occurs at a 40/41 kDa membrane protein very similar or identical to G_i [9,10]. However, an effector system which couples to this G-protein species has not been identified until now.

Recently, Srivastava and Singh [11] reported the co-purification of a GTP-binding protein with the insulin receptor, supporting the notion [12,13] that the insulin receptor is associated with G-proteins which regulate receptor functions. However, controversial findings have been reported concerning this hypothesis. Thus, Davis and McDonald [13] showed that the tyrosine kinase activity of the partially purified insulin receptor from adipose tissue is inhibited by [^{35}S]GTP; however, this finding has not been reproduced by Kellerer et al. [9] using the same tissue. Furthermore, pertussis toxin had no effect on the autophosphorylation and tyrosine kinase activity of the insulin receptor in BC_3H-1 myocytes [8], whereas [^{35}S]GTP decreased insulin-mediated phosphorylation of endogenous proteins in L 6 muscle cells [14]. Interestingly, no activation of insulin receptor kinase by [^{35}S]GTP has been reported so far, although [^{35}S]GTP can induce tyrosine phosphorylation in other systems [15].

In the light of our recent observations of G-protein-mediated insulin signalling in isolated ventricular cardiomyocytes [7] we have now investigated the effects of [^{35}S]GTP on the functional activity of insulin receptors solubilized from these cells. The data suggest that cardiac insulin receptors are coupled to a G-protein which may act as a regulator of tyrosine kinase activity.

Correspondence address: J. Eckel, Diabetes Research Institute, Auf'm Hennekamp 65, D-4000 Düsseldorf 1, Germany. Fax: (49) (211) 338 2603.

Abbreviations: GTP, guanosine 5′-*O*-(3-thiotriphosphate); GDP, guanosine 5′-*O*-(2-thiodiphosphate); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; WGA, wheat germ agglutinin; AMP-PNP, adenyl-5′-yl imidodiphosphate.

2. MATERIALS AND METHODS

2.1. *Chemicals*

[γ-^{32}P]ATP (6,000 Ci/mmol) and [^{35}S]GTP (1,200 Ci/mmol) were purchased from New England Nuclear (Germany). Reagents for SDS-PAGE were supplied by Pharmacia and Sigma (Germany). Triton X-100, poly(Glu:Tyr) 4:1, DTT, [^{35}S]GTP, [^{35}S]GDP, WGA coupled to agarose, phenylmethylsulfonyl fluoride and aprotinin were from Sigma (Germany). Bacitracin, leucine, benzamidine, leupeptin and pepstatin were obtained from Fluka (Switzerland). Pig monocomponent insulin was from Novo (Denmark). All other chemicals were of the highest grade commercially available.

2.2. *Solubilization of insulin receptors and partial purification*

Male Wistar rats fed ad libitum and weighing 280–320 g were used in all experiments. Ca^{2+}-tolerant myocytes were isolated by perfusion of the heart with collagenase as previously described by us [16]. Solubilization and purification of insulin receptors from these cells was performed according to Häring et al. [17] with modifications. 0.7–1 ×

 Published by Elsevier Science Publishers B.V.

10^7 cells were used for each receptor preparation. Cell lysis was achieved by freezing and thawing (three times) the cells in a phosphate buffer (composition NaH_2PO_4 10 mM, sucrose 250 mM, EDTA 5 mM, glycerol 10%, pH 7.5) containing the protease inhibitors benzamidin (10 mM), phenylmethylsulfonyl fluoride (5 mM), leupeptin (2 μM), pepstatin (2 μM), aprotinin (1.2 trypsin inhibiting U/ml), bacitracin (0.01%), and leucine (10 mM). The cell lysate was then centrifuged for 50 min at 20,000 × g and the pellet was solubilized for 90 min in HEPES buffer (composition HEPES 25 mM, Triton X-100 1%, phenylmethylsulfonyl fluoride 2 mM, leupeptin 2 μM, pepstatin 2 μM, aprotinin 0.6 trypsin inhibiting U/ml, pH 7.5). Elution buffer (composition HEPES 25 mM, NaCl 120 mM, KCl 5 mM, $CaCl_2$ 1 mM, $MgSO_4$ 0.8 mM, glycerol 10%) was added to a volume of 10 ml, and this material was centrifuged for 90 min at 100,000 × g. The supernatant was diluted 5 times with elution buffer and applied to a column of WGA coupled to agarose. After washing the column with 50 ml of elution buffer, the bound material was eluted with elution buffer containing 0.3 M *N*-acetylglucosamine. The fraction containing insulin binding activity was frozen in liquid nitrogen and stored at −80°C. Protein was determined using a modification of the Bio-Rad protein assay with BSA as a standard. 15–20 μg glycoprotein was obtained from 10^6 cardiac cells.

2.3. *Autophosphorylation of the partially purified insulin receptor*

3–5 μg of the WGA-purified insulin receptor was incubated for 30 min at 25°C in the absence or presence of the indicated additions in a final volume of 30 μl in phosphorylation buffer (HEPES 25 mM, $MgCl_2$ 10 mM, $MnCl_2$ 5 mM, Na_3VO_4 0.3 mM, pH 7.5). The reaction was initiated by adding 10 μCi of [γ-^{32}P]ATP (final concentration 28 μM) and conducted for 10 min. The incubation was terminated by addition of 10 μl of Laemmli [18] sample buffer (250 mM Tris, 8% SDS, 20% glycerol, and 0.04% Bromphenol blue), containing 1 mM ATP and 200 mM DTT, and boiling for 10 min. Phosphoproteins were analyzed by SDS-PAGE using 4% stacking and 7% resolving gels. The gels were subjected to autoradiography using Kodak X-Omat AR film and intensifying screens. Autoradiographs were quantified by laser-scanning densitometry. Significance of reported differences was evaluated by using the null hypothesis and t statistics for paired data.

2.4. *Assay of tyrosine kinase activity*

Aliquots (0.6–1 μg) of the partially purified insulin receptor were preincubated in phosphorylation buffer (final volume 65 μl) for 30 min at 25°C in the absence or presence of the indicated additions. Incubation was then continued for 30 min in the absence or presence of insulin (3.2×10^{-7} M). The phosphorylation reaction was started by addition of [γ-^{32}P]ATP (6 μCi, final concentration 50 μM) and the exogenous substrate poly(Glu:Tyr) (0.2 mg/ml). The reaction was allowed to proceed for 20 min and was terminated by addition of 50 mM unlabelled ATP and applying triplicate aliquots (10 μl) onto Whatman P81 filter papers. The filter papers were extensively washed in 10% trichloroacetic acid containing 10 mM sodium pyrophosphate, dried, and radioactivity counted by liquid scintillation. Non-specifically bound radioactivity was defined as that bound to the filters in the absence of poly(Glu:Tyr) and was subtracted from that bound in the presence of poly(Glu:Tyr).

2.5. *[^{35}S]GTP binding assay*

Binding of [^{35}S]GTP to WGA-purified proteins was determined by incubating 0.5–1 μg glycoprotein in phosphorylation buffer at 25°C in a final volume of 100 μl. After preincubating the protein solution for 30 min in the absence or presence of insulin, the binding reaction was initiated by the addition of 1 μCi [^{35}S]GTP (final concentration 10 nM) and terminated at the indicated times by the addition of 2 ml of ice-cold stop solution (Tris 20 mM, NaCl 100 mM, $MgCl_2$ 25 mM, pH 8.0) followed by rapid filtration through B85 nitrocellulose filters (Schleicher & Schuell). The filters were washed with 7 ml stop solution, dried and placed into 10 ml of scintillation fluid. Radioactivity was then determined in a Beckman LS 6000 IC scintillation counter. All experiments were performed in triplicate. Non-specific binding was determined in parallel incubations in the presence of 100 μM unlabelled GTP. All binding data reported represent specific binding defined as the difference between total and non-specific binding.

3. RESULTS

Preincubation of the partially purified insulin receptor with [^{35}S]GTP resulted in an increased incorporation of ^{32}P into the 95 kDa β-subunit of the insulin receptor (Fig. 1). As presented in Table I, the nucleotide increases autophosphorylation about two times, reaching about one half of the stimulatory action of insulin obtained at maximal concentrations of the hormone. It may be argued that the effect of [^{35}S]GTP is related to elevated ATP concentrations during the incubation period due to an inhibition of nucleotidase activity. However, under all incubation conditions no significant alteration of the total ATP concentration (50 μM) could be detected using the firefly luciferase assay [7].

In order to elucidate if the [^{35}S]GTP-induced increase in autophosphorylation of the insulin receptor leads to an increased tyrosine kinase activity, we have studied

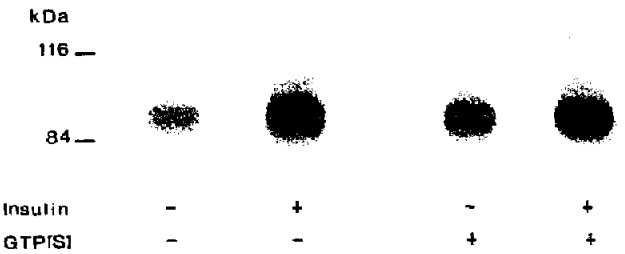

Fig. 1. Effect of [^{35}S]GTP on insulin receptor autophosphorylation. 3–5 μg of partially purified insulin receptor were preincubated for 30 min in the absence or presence of [^{35}S]GTP (200 μM). Incubation was then continued for an additional 30 min in the absence or presence of insulin (3.5×10^{-7} M). The phosphorylation reaction was initiated by adding 28 μM [^{32}P]ATP and conducted for 10 min. Phosphoproteins were separated by SDS-PAGE and subjected to autoradiography. A representative autoradiogram of three separate experiments is shown.

Table I

Effect of insulin and [^{35}S]GTP on insulin receptor β-subunit autophosphorylation

Incubation conditions	β-subunit autophosphorylation (% of maximum)
Basal	21.5 ± 3.9
Insulin	88.7 ± 11.2
[^{35}S]GTP	38.7 ± 8.9*
Insulin + [^{35}S]GTP	97.5 ± 2.5

Experimental conditions are described in the legend to Fig. 1. The relative amount of β-subunit autophosphorylation was determined by laser-scanning densitometry of individual autoradiograms. Mean values (% of maximum) of three separate experiments (±S.E.M.) are shown.

*Significantly different from basal with $P < 0.05$.

the effect of the nucleotide on insulin stimulated phosphorylation of the synthetic substrate poly(Glu:Tyr), reflecting the intrinsic tyrosine kinase activity of the receptor. As presented in Fig. 2, preincubation with [^{35}S]GTP (100 μM) fully mimicked the stimulatory action of insulin on poly(Glu:Tyr) phosphorylation. Under these conditions no significant effect of the hormone on poly(Glu:Tyr) phosphorylation could be detected, suggesting that [^{35}S]GTP stimulates the insulin receptor kinase in an insulin-like fashion. In contrast, the pyrimidine triphosphate, CTP, did not influence basal and insulin stimulated poly(Glu:Tyr) phosphorylation.

The effect of [^{35}S]GTP was dose-dependent, with a maximal effect of the nucleotide at 100 μM (Fig. 3). The stimulatory action of [^{35}S]GTP persisted even up to 1 mM. [^{35}S]GDP, an antagonist of G-protein-mediated functions, was without effect on poly(Glu:Tyr) phosphorylation. To further characterize the mechanism of GTP action, the ATP concentration was varied in the range of 12.5 μM up to 200 μM. Lineweaver–Burk analysis of the phosphorylation of the synthetic polypeptide in the absence or presence of [^{35}S]GTP (100 μM) showed that the K_m of the partially purified insulin receptor for poly(Glu:Tyr) phosphorylation was not significantly affected by [^{35}S]GTP (43 and 54.1 μM for control and [^{35}S]GTP, respectively). However, the V_{max} increased from 1.88 to 3.46 pmol/μg protein × 20 min when preincubating the receptor with [^{35}S]GTP. This agrees with the well-established effect of insulin on insulin receptor tyrosine kinase activity, which is exclusively due to an increase in V_{max} [19].

The effects of [^{35}S]GTP on the function of solubilized insulin receptors may involve either a direct interaction

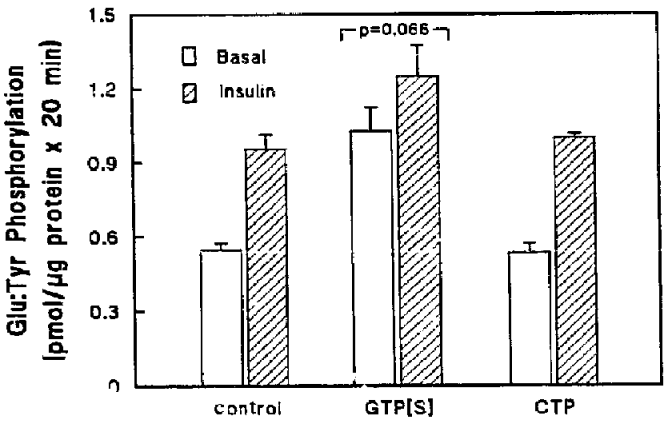

Fig. 2. Effect of [^{35}S]GTP and CTP on exogenous substrate protein kinase activity of the WGA-agarose-purified insulin receptor. Aliquots (0.6–1 μg) of the glycoprotein fraction were preincubated for 30 min in the absence (control) or presence of [^{35}S]GTP (100 μM) or CTP (100 μM). Incubation was then continued for 30 min in the absence or presence of insulin (3.2×10^{-7} M). Poly(Glu:Tyr) (0.2 mg/ml) phosphorylation was then determined using 50 μM ATP as described in section 2. Data are means ± S.E.M. obtained from three independent receptor preparations.

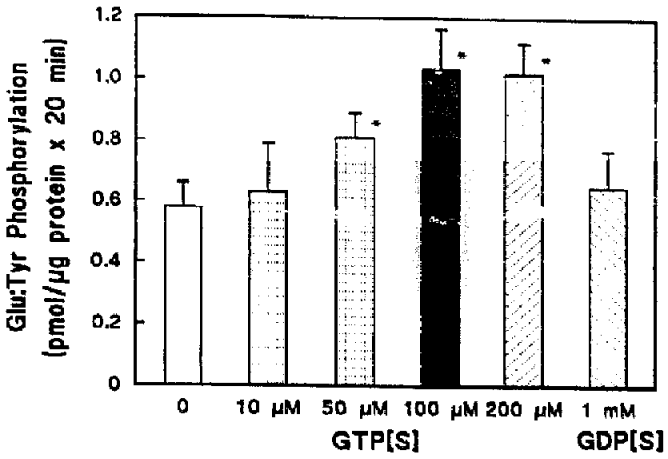

Fig. 3. Effect of [^{35}S]GTP concentration on exogenous substrate protein kinase activity. Partially purified insulin receptor was preincubated with the indicated concentrations of guanosine nucleotides for 30 min. Kinase activity was then determined as outlined in Fig. 2. Values are means ± S.E.M. (n = 3–4). *Significantly different from control with $P < 0.05$.

of the receptor with the nucleotide or a GTP-binding protein closely coupled to the receptor. Since hormone receptors which couple to G-proteins increase the binding of GTP, we have studied the effect of insulin on the time–course of [^{35}S]GTP binding to WGA-purified proteins. As shown in Fig. 4 (upper panel), insulin significantly enhanced [^{35}S]GTP binding with a most prominent effect (196% of control) at early time points. When ATP was removed from the incubation medium (Fig. 4, middle panel), the effect of insulin was no longer detectable. In order to elucidate if autophosphorylation of the insulin receptor β-subunit may be essential for coupling to the G-protein system, a similar experiment was performed, substituting ATP by the non-hydrolyzable analogue, AMP–PNP. As presented in Fig. 4 (lower panel), the effect of ATP can be fully mimicked by AMP–PNP, suggesting that the insulin receptor interacts with the G-protein in a tyrosine kinase-independent fashion.

4. DISCUSSION

Originally it was thought that coupling to GTP-binding proteins is exclusively limited to hormone receptors with several membrane spanning domains [20]. However, more recent studies suggest that single-spanning receptors, like epidermal growth factor receptor [21], insulin-like growth factor-I [22] and -II [23] receptor, and insulin receptor, may also be coupled to G-proteins. In the latter case, the situation may be even more complex. Thus, GTP-binding proteins have been suggested to be involved in insulin signalling both at the receptor [9,13,24] and the post-receptor level [7,14,25]. The identity of the G-proteins and the functional implications, however, remain poorly understood.

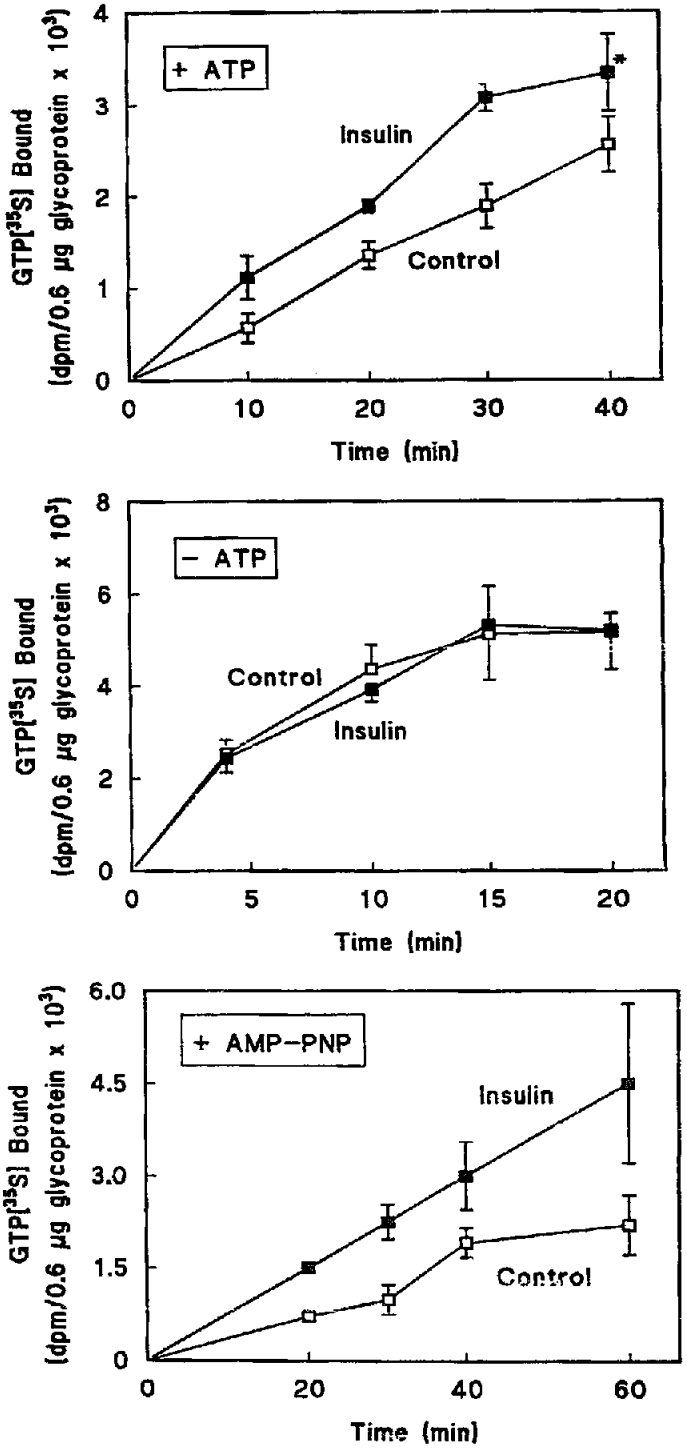

Fig. 4. Effect of insulin on [^{35}S]GTP binding to WGA-purified proteins. Glycoprotein (0.5–1 µg) was incubated for 30 min at 25°C in the absence (□) or presence (■) of insulin (1 µM). 1 µCi [^{35}S]GTP (final concentration 10 nM) was then added and binding was measured as detailed in section 2. All data have been corrected for non-specific binding. (Upper panel) All incubations were performed in the presence of ATP 30 µM). (Middle panel) No adenosine nucleotide present. (Lower panel) All incubations were performed in the presence of AMP-PNP (500 µM). Values are means ± S.E.M. of 3–4 receptor preparations. *Not significantly different from control with $P > 0.05$.

In the present investigation we have used insulin receptors solubilized from ventricular cardiomyocytes in order to test the hypothesis that the insulin receptor is closely coupled to a G-protein, which may operate as a regulator of insulin receptor functions. A major finding is the observation that [^{35}S]GTP increases autophosphorylation, and most probably thereby activates the tyrosine kinase activity of the receptor preparation. Several lines of evidence suggest that the nucleotide mimicks the effect of insulin on the tyrosine kinase activity of the isolated insulin receptor. First, [^{35}S]GTP increased poly(Glu:Tyr) phosphorylation to the level observed with insulin; second, no additive effect of the hormone could be detected; and third, [^{35}S]GTP increased the V_{max} of the kinase in agreement with the effect of insulin [19].

In contrast to our findings, Davis and McDonald [13], using adipocyte insulin receptors, recently reported on an inhibition of β-subunit autophosphorylation and a reduction of tyrosine kinase activity by [^{35}S]GTP at high concentrations of the nucleotide. Furthermore, Kelleret et al. [9] described an inhibition of insulin-stimulated receptor kinase activity by [^{35}S]GTP in adipocyte plasma membrane preparations. The apparent discrepancy between these studies and our results may be explained by very recent observations from McDonald's group, published in abstract form [26], in which it was shown that placenta insulin receptors are associated with a 67 and a 41 kDa G-protein. Removal of the 67 kDa G-protein increases the tyrosine kinase activity, whereas the 41 kDa G-protein is suggested to be responsible for insulin sensitivity of the kinase. Since the purification protocol may affect the amount and the nature of G-proteins associated with the insulin receptor, differing results concerning kinase regulation are not surprising. Preliminary photolabeling experiments in our laboratory have shown that the purified insulin receptor preparation indeed contains a variety of G-proteins, but only one species is activated by insulin. In light of the above mentioned observations [26] our data would be compatible with the dissociation of a G-protein from the receptor upon addition of [^{35}S]GTP, finally leading to a kinase activation.

The assumption that [^{35}S]GTP binds to a G-protein and not to the insulin receptor itself is supported by earlier work showing that GTP has no effect on highly purified insulin receptor preparations [27]. Additional evidence for the functional relationship between the WGA-purified insulin receptor and a G-protein is obtained from the studies on [^{35}S]GTP binding. Insulin increased [^{35}S]GTP binding under conditions which are required for insulin signalling, suggesting that the insulin receptor is closely coupled, most probably associated with a G-protein. Consistently, the co-purification of G-proteins with the insulin receptor has been reported by several groups [11,26].

Insulin receptor/G-protein coupling was observed in

the presence of ATP or AMP-PNP, but could not be detected in the absence of these nucleotides. This finding suggests that the communication between the insulin receptor and the G-protein system does not involve autophosphorylation of the insulin receptor. However, insulin signalling appears to be dependent on the presence of ATP, most probably on the binding of the nucleotide to the β-subunit of the insulin receptor. The latter finding agrees well with a recent report by Maddux and Goldfine [28] showing that insulin plus ATP may induce a conformational change in the β-subunit without inducing receptor autophosphorylation. In light of our findings it may be speculated that conformational changes of the insulin receptor result in a non-covalent interaction with G-proteins. Furthermore, in this model of non-covalent insulin receptor/G-protein coupling, a G-protein-mediated activation of the insulin receptor tyrosine kinase by insulin would represent a putative pathway of insulin signalling in target cells. This hypothesis, although still highly speculative, needs further attention.

Taken together, the present study supports the notion that the insulin receptor interacts with a G-protein and that this interaction modulates receptor functions.

Acknowledgements: This work was supported by the Ministerium für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, the Bundesministerium für Gesundheit, and the Deutsche Forschungsgemeinschaft (SFB 351, C 2).

REFERENCES

[1] Heyworth, C.M. and Houslay, M.D. (1983) Biochem. J. 214, 547–552.
[2] Goren, H.J., Northup, J.K. and Hollenberg, M.D. (1985) Can. J. Physiol. Pharmacol. 63, 1017–1022.
[3] Luttrell, L.M., Hewlett, E.L., Romero, G. and Rogal, A.D. (1988) J. Biol. Chem. 263, 6134–6141.
[4] Rothenberg, P.L. and Kahn, C.R. (1988) J. Biol. Chem. 263, 15546–15552.
[5] Obermaier-Kusser, B., Mühlbacher, C., Mushack, J., Rattenhuber, E., Fehlmann, M. and Häring, H.U. (1988) Biochem. J. 256, 515–520.
[6] Ciaraldi, T.P. and Maisel, A. (1989) Biochem. J. 264, 389–396.
[7] Eckel, J., Gerlach-Eskuchen, E. and Reinauer, H. (1990) Biochem. J. 272, 691–696.
[8] Luttrell, L., Kilgour, E., Larner, J. and Romero, G. (1990) J. Biol. Chem. 265, 16873–16879.
[9] Kellerer, M., Obermaier-Kusser, B., Pröfrock, A., Schleicher, E., Seffer, E., Mushack, J., Ermel, B. and Häring, H.U. (1991) Biochem. J. 276, 103–108.
[10] Pröfrock, A., Schnefel, S. and Sch[illegible], I. (1991) Biochem. Biophys. Res. Commun. 175, 380–386.
[11] Srivastava, S.K. and Singh, U.S. (1990) Biochem. Biophys. Res. Commun. 173, 501–506.
[12] Mortensen, E.R., Drachman, J. and Guidotti, G. (1992) Biochem. J. 281, 735–743.
[13] Davis, H.W. and McDonald, J.M. (1990) Biochem. J. 270, 401–407.
[14] Burdett, E., Mills, G.B. and Klip, A. (1990) Am. J. Physiol. 258, C99–C108.
[15] Nasmith, P.E., Mills, G.B. and Grinstein, S. (1989) Biochem. J. 257, 893–897.
[16] Eckel, J., Pandalis, G. and Reinauer, H. (1983) Biochem. J. 212, 385–392.
[17] Häring, H.U., Machicao, F., Kirsch, D., Rinninger, F., Hölzl, J., Eckel, J. and Bachmann, W. (1984) FEBS Lett. 176, 229–234.
[18] Laemmli, U.K. (1970) Nature 227, 680–685.
[19] Shoelson, S.E. and Kahn, C.R., in: Insulin Action (Draznin, B., Melmed, S. and LeRoith, D., eds.) pp. 23–33, Liss Inc., New York, 1989.
[20] Lefkowitz, R.J. and Caron, M.G. (1988) J. Biol. Chem. 263, 4993–4996.
[21] Pike, L.J. and Eakes, A.T. (1987) J. Biol. Chem. 262, 1644–1651.
[22] Nishimoto, I., Ogata, E. and Kojima, I. (1987) Biochem. Biophys. Res. Commun. 148, 403–411.
[23] Nishimoto, I., Murayama, Y., Katada, T., Ui, M. and Ogata, E. (1989) J. Biol. Chem. 264, 14029–14038.
[24] Müller-Wieland, D., White, M.F., Behnke, B., Gebhardt, A., Neumann, S., Krone, W. and Kahn, C.R. (1991) Biochem. Biophys. Res. Commun. 181, 1479–1485.
[25] Baldini, G., Hohman, R., Charron, M. and Lodish, H.F. (1991) J. Biol. Chem. 266, 4037–4040.
[26] Jo, H., Cha, B.Y. and McDonald, J.M. (1992) Diabetes 41, suppl. 1, abstract 32.
[27] Ridge, K.D., Hofmann, K. and Finn, F.M. (1988) Proc. Natl. Acad. Sci. USA 85, 9489–9493.
[28] Maddux, B.A. and Goldfine, I.D. (1991) J. Biol. Chem. 266, 6731–6736.